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(71) Applicant (for all designated States except US): LYNXVALE LIMITED [GB/GB]; The Old Schools, Cambridge CB2 1TS (GB).

(72) Inventor; and
(75) Inventor/Applicant (for US only): CLARK, Michael, Ronald [GB/GB]; 124 Richmond Road, Cambridge CB4 3PT (GB).

(74) Agent: O'BRIEN, Caroline; Mewburn Ellis, York House, 23 Kingsway, London WC2B 6HP (GB).

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(54) Title: BINDING MOLECULES CONTAINING AT LEAST AN IMMUNOGLOBULIN CONSTANT DOMAIN WITH MODIFIED ALLOTYPIC DETERMINANT

(57) Abstract

The application provides a binding molecule which has a first amino acid sequence comprising a domain with an ability to bind to a target molecule; and a second amino acid sequence substantially homologous to part or all of the constant region of a human immunoglobulin heavy chain, but which differs in an allotypic determinant; wherein the difference in the allotypic determinant results in said binding molecule having an improved effector function as compared to a binding molecule having the first amino acid sequence and part or all of the constant region of the immunoglobulin heavy chain. Also provided is a method for making a binding molecule which has a first amino acid sequence comprising a domain with an ability to bind to a target molecule and a second amino acid sequence comprising part or all of a human immunoglobulin heavy chain having an allotypic determinant of a sequence associated with a desired effector function.

1

Binding molecules containing at least an immunoglobulin constant domain with modified allotypic determinant

The present invention relates to binding molecules.

Owing to their high specificity for a given antigen,

5   antibodies and particularly monoclonal antibodies (Kohler,

G. and Milstein C., 1975 Nature 256:495) represented a

significant technical break-through with important

consequences scientifically, commercially and

therapeutically.

10   Monoclonal antibodies are made by establishing an

immortal cell line which is derived from a single

immunoglobulin producing cell secreting one form of a

biologically functional antibody molecule with a particular

specificity.

15   Owing to their specificity, the therapeutic

applications of monoclonal antibodies hold great promise

for the treatment of a wide range of diseases (Clinical

Applications of Monoclonal Antibodies, edited by E. S.

Lennox. British Medical Bulletin 1984, publishers

20   Churchill Livingstone). Structurally, the simplest

antibody (IgG) comprises four polypeptide chains, two heavy

(H) chains and two light (L) chains inter-connected by

disulphide bonds (see figure 1). The light chains are of

two types, either kappa or lambda. Each of the H and L

25   chains has a region of low sequence variability, the

constant region (C), differences therein giving rise to

allotypes and a region of high sequence variability, the

variable region (V) differences therein giving rise to
idiotypes. The antibody has a tail region (the Fc region)
which comprises two or three domains of the C regions of
the two H chains. The tail region is responsible for

5   interactions with the effector systems through binding and
activation of complement and Fc receptors.

The antibody also has two arms (the Fab region) each
of which has a V_L and a V_H region associated with each other
and each connected to a C region domain, C_L and CH_1

10 respectively. In addition there is a hinge region between
the CH_1 and CH_2 domains. Typically the C region of an IgG H
chain consists of CH_1 hinge CH_2 and CH_3. It is this pair of
V regions (V_L and V_H) that differ from one antibody to
another, and which together are responsible for recognising

15 the antigen. In even more detail, each V region is made up
from three complementarity determining regions (CDR)
separated by four framework regions (FR). The CDRs are the
most variable part of the variable regions, and they
perform the critical antigen binding function. The CDR

20 regions are derived from many potential germ line sequences
via a complex process involving recombination, mutation and
selection. It has been shown that the function of binding
antigens can be performed by fragments of a whole antibody
and this is well documented in the art.

25 There are a range of different immunoglobulins IgG,

3

IgM, IgA, IgD, IgE, known as isotypes. Of these, IgG is
most commonly used therapeutically. It exists as isotypic
sub-classes IgG1, IgG2, IgG3 and IgG4.

The amino acid sequence for the constant region is
reasonably conserved. Nevertheless sequence variation does
exist and there are 24 recognised allotypes of human
immunoglobulin distributed between the different isotypes
as follows:

        IgG1   x   4

        IgG2   x   1

        IgG3   x 13

        IgA2   x   2

        IgE   x   1

        Kappa x   3

The allotypes represent alternative amino acid
substitutions found at discrete sites in the protein
sequence. These different allotypic determinants are found
in different combinations within given allelic forms of
genes, but not all possible combinations which
theoretically might exist are in practice observed.

For example, four different allotypes of IgG1 can be
seen (ie distinguished) by the immune system. These are
G1m 1, 2, 3 and 17. Alternatively, combinations thereof,
such as G1m (1, 17), can also be distinguished.

Antisera can be raised in other non-human species

which can see the alternative isoallotypes provided that the antibody is purified away from the other human isotypes. Such isoallotypes for which such an antisera exists have been called non-allotypes and given the designation for example, nG1m(1) which is the isoallotype of G1m(1). Thus, although a human isoallotype should not be immunogenic in humans, it can still potentially be recognized in a different species.

Of the above mentioned different allotypes of IgG1, three common allelic forms of human IgG1 occur with different frequencies within different racial groups, namely G1m (3), G1m (1, 17), and G1m (1, 2, 17) based upon their reactivities with human antisera directed against the determinants G1m 1, 2, 3 and 17.

For the purposes of clarity the sequences around the allotypic sites G1m (1) (2) and (17) are shown below for each isotype.

<u>Site (1)</u>

<u>355</u>	<u>356</u>	<u>357</u>	<u>358</u>	
Arg	Asp or Glu	Glu	Leu or Met	IgG1
Arg	Glu	Glu	Met	IgG2
Arg	Glu	Glu	Met	IgG3
Gln	Glu	Glu	Met	IgG4

Thus, at site (1), IgG1 may exist as several allotypes depending on whether aspartic acid or glutamic acid at

position 356, or leucine or methionine at position 358 are
present.

Site 2

430	431	432	
Glu	Gly or Ala	Leu	IgG1
Glu	Ala	Leu	IgG2
Glu	Ala	Leu	IgG3
Glu	Ala	Leu	IgG4

Thus, at site (2), IgG1 may exist as either of two
allotypes depending on whether glycine or alanine is
present at position 431.

Site (17)/(3)

Sites (3) and (17) are alternative substitutions at the
same site.

213	214	215	
Lys	Lys or Arg	Val	IgG1
Lys	Thr	Val	IgG2
Lys	Arg	Val	IgG3
Lys	Arg	Val	IgG4

Thus, at site (17)/(3), IgG1 may exist as either of
two allotypes depending on whether lysine or arginine is
present. The allotypes (17) and (3) cannot co-exist as
they represent alternative substitutions at the same
position.

Antibodies are generally raised in animals,

particularly rodents for monoclonal antibodies and
therefore the immunoglobulins produced, have characteristic
features specific to that species. The repeated
administration of these foreign rodent proteins for
therapeutic purposes to human patients can lead to harmful
hypersensitivity reactions. In part, this problem has been
overcome in recent years by using the techniques of
recombinant DNA technology to 'humanise' non-human
antibodies. Basically this involves creating a synthetic
antibody having a mainly human sequence but retaining the
rodent sequences determining the antibody specificity eg
rodent CDRs. However problems still remain because of
allotypic differences when compared with the patients own
equivalent antibodies. Recently the present applicants
have proposed that one way around this problem is to
eliminate the allotypic variation from the constant region
(see publication No. WO92/16562 corresponding to
Application No. PCT/GB92/00445).

Going on from this earlier work the present applicants
have now made the surprising discovery that allotypic
polymorphism is to a certain extent responsible for
differences in observed effector functions. Previously,
differences in effector function was thought to be
determined only by isotype and subclass (eg IgG1 versus
IgG4) within a given isotype.

7

Antibodies exert their cytolytic effects via two main
pathways: complement-mediated cytotoxicity (CMC) or
antibody-dependent cell-mediated cytotoxicity (ADCC).

In CMC, there will be fixation of the first protein
5   component (C1 via C1q) of the complement cascade leading to
cleavage of C3, activation of the lytic pathway of the
complement system, and to cytolysis of the foreign cells.
In addition, the activation of C3 causes platelet
aggregation, accumulation of neutrophils, and the formation
10   of an inflammatory exudate. As a result of this, bystander
tissue cells can be damaged even though they are not the
immediate targets of the antibody recognition.

In ADCC, the antibody coated target cells are
recognised by cytotoxic cells (eg monocytes and
15   polymorphonuclear cells) having Fc receptors. Binding will
occur between the target cells and cytotoxic cells via the
antibody-Fc receptor bridge and lysis of the target cells
will follow.

Different isotypes differ with regard to the effector
20   systems with which they interact. Therefore, not all
isotypes are able to activate complement equally well and
Fc receptors (FcR) also show specificity of binding eg only
a certain group of FcRs bind IgA, another group IgE and
another group IgG. FcRs also occur as different classes
25   and subclasses eg FcγRI, FcγRII and FcγIII for those

8

binding IgG (reviewed by van de Wintel and Capel 1993

Immunol. Today 14, 215-221).

Even within an antibody class, there are subclass

differences in relation to effector function and these

5   differences can be broadly stated. Thus human IgG4 does

not seem to activate complement at all and binds FcγRI and

FcγRII poorly. IgG1 and IgG3 are active in both complement

activation and in binding to the three classes of receptor

FcγRI, FcγRII and FcγRIII. IgG2 can activate complement

10   under some circumstances (eg high antigen density) but does

not bind to most FcγRs (Bruggeman et al., 1987 J. Exp. Med.

166, 1351-1361; Riechmann et al., 1988 Nature 332, 323-327;

Greenwood, Clark and Waldmann 1993 Eur. J. Immunol. 23,

1098-1104).

15   Some receptors exist in a polymorphic form (ie as

alleles) and there is an allelic form of the FcγRII

receptor that can bind IgG2.

The subclasses 1-4 of IgG are very homologous in amino

acid sequence (>90%), particularly if the hinge regions

20   (which are not highly conserved) are treated separately.

If the constant regions are very similar with only a few

amino acid differences, yet the subclasses exhibit

different effector functions, some or all of the amino acid

differences may be responsible for moderating the observed

25   functions. Experiments have been carried out in which the

9

residues which differ between subclasses are mutated or
exchanged to discover which of them are responsible for the
observed differences (reviewed in Greenwood and Clark 1993
Protein Engineering of antibody molecules for prophylactic
5   and therapeutic applications in man (ed. Clark, M.) Publ.
Academic Titles, UK (1993) p 85-100 Effector functions of
matched sets of recombinant IgG subclass antibodies).

The present applicants have now made the surprising
discovery that an antibody's allotype can determine its
10   effector function. This enables the design of therapeutic
molecules having a binding domain linked to a human
immunoglobulin heavy chain constant region having a
particularly desired effector function. Prior to the
present applicants disclosure this has not been possible.

15   The present invention provides a binding molecule
which has a first amino acid sequence comprising a domain
with an ability to bind to a target molecule; and a second
amino acid sequence substantially homologous to part or all
of the constant region of a human immunoglobulin heavy
20   chain, but which differs in an allotypic determinant;
wherein the difference in the allotypic determinant results
in said binding molecule having an improved effector
function as compared to a binding molecule having the first
amino acid sequence and part or all of the constant region
25   of the immunoglobulin heavy chain.

Also provided is a method for making a binding
molecule which has a first amino acid sequence comprising a
domain with an ability to bind to a target molecule and a
second amino acid sequence comprising part or all of a

5   human immunoglobulin heavy chain having an allotypic
determinant of a sequence associated with a desired
effector function which method comprises: (a) examining one
or more sequences, each comprising part or all of a human
immunoglobulin heavy chain and obtaining said second amino

10   acid sequence by either (i) selecting one of said one or
more sequences on the basis of it comprising a said
allotypic determinant; or (ii) by selecting one of said one
or more sequences on the basis of it comprising a sequence
suitable for altering to a said allotypic determinant and

15   altering the sequence to make a said second amino acid
sequence; (b) obtaining a said first amino acid sequence;
(c) creating the binding molecule by either (i) chemically
linking said first and second amino acid sequences; or (ii)
making a nucleotide construct encoding said first and

20   second amino acid sequences, transforming a suitable host
cell with the nucleotide construct, and causing said host
cell to express the binding molecule in the form of a
fusion polypeptide.

The domain with an ability to bind to a target

25   molecule may derive from any molecule with specificity for

11

another molecule. Thus the domain may derive from an enzyme, a hormone, a receptor (cell-bound or circulating) a cytokine or an antigen or antibody.

Of particular interest are the cytokines IL-2,IL-4, IL-5, IL-8, IL-12,IL-13 and IL-15 which are involved in the modulation of the immune response. These interleukins are referenced by Taniguchi et al (1983) Nature 302: 305 (IL-2), Yokota et al (1986) PNAS 83: 5894 (IL-4), Campbell et al (1987) PNAS 84: 6629 (IL-5), Yoshimura et al (1987) PNAS 84: 9233 (IL-8), Gubler et al (1991) PNAS 88, 4143 (IL-12), McKenzie et al (1993) PNAS 90: 3735 (IL-13), Grabstein et al (1994) Science 264: 965 (IL-15).

Also of interest are the soluble forms of the OX-40 receptor (Latza, U., et al 1994 Eur. J. Immunol. 24 : 677) and of the OX-40 ligand (Godfrey W.K., et al 1994 J. Exp. Med. 180 : 757). The human OX-40 receptor is expressed primarily on activated CD4+ T cells.

Alternatively the domain may derive from an antigen.

Alternatively the domain may be part or all of an antibody or a derivative thereof. Thus a binding molecule according to the present invention may provide a rodent originating antibody binding domain and a human immunoglobulin heavy chain having a particular allotypic determinant which predisposes the binding molecule to a particular effector function.

12

The above refers to an improved effector function.
This means that the binding molecule may have an effector
function of a different type to that of a binding molecule
having the first amino acid sequence and part or all of the

5   constant region of the immunoglobulin heavy chain.
Alternatively, the effector function of the binding
molecule may be of the same type as a binding molecule
having the first amino acid sequence and part or all of the
constant region of the immunoglobulin heavy chain, but the

10   effector function being present to a lesser or greater
degree. The improvement is essentially that the effector
function is more appropriate to the particular utility of
the binding molecule.

Thus in situations where it is important to maximise

15   the binding molecules activities in complement lysis it may
be appropriate to have an arginine residue at position 214
in the CH_2 domain.

Sequences for human immunoglobulin heavy chains are
known and published. The sequence information can be

20   obtained from the SwissProt and PIR databases using
Lasergene software (DNAStar Limited, London UK) under
accession numbers A93433, B90563, A90564, B91668, A91723
and A02146 for human Igγ-1 chain C region, A93906, A92809,
A90752, A93132, A02148 for human Igγ-2 chain C region,

25   A90933, A90249, A02150 for human Igγ-4 chain C region,

13

A92249, A91662, A02171 for human Igα-1 chain C region,
A93828, A93829 and A02172 for human Igα-2 chain C region
and A23511 for human Igγ-3 chain C region.

The effector functions associated with the various
allotypes of the human immunoglobulin heavy chains may be
established in accordance with the general teachings
herein, as exemplified by the present applicants
establishment that it is the arginine at position 214 which
is necessary for lysis in the aglycosylated IgG1 antibodies
and that having in the alternative a lysine or a threonine
residue at this position does not give detectable lytic
activity.

Then one may either select a human immunoglobulin
heavy chain having the appropriate amino acid residues
which predispose to a particular desired effector function
or modify a human immunoglobulin heavy chain in order to
make a heavy chain which has the appropriate amino acid
residues which predispose to a particular effector
function.

The heavy chain may then be coupled to an amino acid
sequence comprising a binding domain at the protein level
ie by chemical coupling methods. A variety of methods are
available to the skilled person to couple antibody
molecules to other moieties such as radio-nucleotides,
toxins, enzymes, cytotoxic drugs and other antibody

14

molecules. They include covalent attachment to exposed
tyrosine residues, or to the ε-amino acid side chains of
aspartic and glutamic acids. Alternatively, sulphydryl
groups generated by the chemical reduction of cystine

5   residues have been used to cross-link antibody domains
(Rhind, S K (1990) EP 0385601 Cross-linked antibodies and
processes for their preparation). Finally, chemical
modification of carbohydrate groups has been used to
generate reactive groups for cross-linking purposes. These

10   methods are standard techniques available to those skilled
in the art.

Alternatively it may be more appropriate to use
recombinant techniques to express the binding molecule in
the form of a fusion protein. Broadly speaking this would

15   involve making a nucleotide construct encoding the first
and second amino acid sequences, using the construct to
transform a suitable host cell and then causing the host
cell to express the binding molecule. This may be done in
accordance with standard methodologies and in general,

20   reference may be made to "Molecular Cloning" A Laboratory
Manual, eds. Sambrook, Fritsch and Maniatis, Cold Spring
Harbor Laboratory Press 1989.

Thus, the domain may comprise an antibody binding
domain. The antibody binding domain may be non-human. The

25   antibody binding domain may be of rodent origin.

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The domain may comprise part or all of a cytokine.
The cytokine may be selected from IL-2, IL-4, IL-5, IL-8,
IL-12, IL-13 and IL-15. In particular the cytokine may be
IL-12.

5   The domain may comprise part or all of a soluble form
of a cell bound ligand or a cell bound receptor. The cell
bound receptor may comprise the OX-40 receptor. The cell
bound ligand may comprise the OX-40 ligand.

The human immunoglobulin heavy chain may be of the
10   isotype IgG1. The difference in an allotypic determinant
may be in the CH_1 domain. The difference in an allotypic
determinant may comprise the presence or absence of
arginine at position 214. Where arginine is absent from
position 214, lysine or threonine may instead be present.
15   The difference in an allotypic determinant may be in the CH_2
domain. The difference in an allotypic determinant may
comprise the presence or absence of asparagine at position
297. Where asparagine is absent from position 297, alanine
may instead be present.

20   The second amino acid sequence may differ from the
part or all of the constant region of an immunoglobulin
heavy chain in more than one allotypic determinant. The
differences may be in both the CH_1 and CH_2 domains. An
amino acid other than arginine (eg lysine or threonine) may
25   be present at position 214 of the CH_1 domain and an amino

acid other than asparagine (eg alanine) may be present at position 297 of the CH_2 domain.

The present invention also provides a pharmaceutical preparation which comprises a binding molecule as above.

The present invention also provides a reagent which comprises a binding molecule as above.

The present invention also provides a method of treating a patient which comprises administering a pharmaceutical as above.

Also provided is use of a binding molecule as above in the preparation of a pharmaceutical to modify an immune response.

Also provided is a packet comprising a pharmaceutical or reagent as provided above along with instructions specifying use thereof.

The present invention also provides a recombinant construct which comprises a nucleotide sequence coding for a binding molecule as above. Also provided are cloning and expression vectors which comprise a recombinant construct as above, along with host cells comprising such vectors and transgenic animals carrying such recombinant constructs.

In order that the present invention is more fully understood, embodiments will be described in more detail by way of example only and not by way of limitation with

reference to the figures listed below.

Figure 1 illustrates the structure of an IgG antibody (as discussed earlier).

Figure 2 shows the M13TG131 cloning vector containing the human gamma-1 constant region and showing cloning sites and modified polylinker.

Figures 3 shows a complement dependent assay for the killing of autologous human peripheral blood lymphocytes with five CAMPATH-1 (CDw52) specific antibody constructs, TF57-19, MTF121, MTF123, MTF133 and MTF134. The degree of lysis was calculated from the % specific release of the radioisotope ^{51}Cr. The results for the three allotypes of human $\gamma 1$ agreed with earlier findings, ie there is no significant difference between TF57-19, MTF121 and MTF123 when assayed under conditions for optimal lysis. Aglycosylated mutants of TF57-19 and MTF121 were constructed by mutating the Asn 297->Ala giving rise to MTF133 and MTF134 respectively. As expected, these new constructs had a reduced activity in this assay, but unexpectedly there was a significant difference between them, as MTF134 still gave some residual lysis. As these two constructs differ only by allotypic residues in the CH_1 and CH_3 domains, being identical in the hinge and CH_2 regions, this is an interesting observation.

Figure 4 shows results of an experiment following on

18

from the results given in Figure 3. A new construct BTF186
was prepared and compared with the previous constructs.
Note that the results are expressed as ^{51}Cr released in cpm
and have not been converted to % specific release. The

5   results for the 5 original constructs agree very well with
the results in Figure 3 and show the reproducibility in the
assay. Quite clearly, construct BTF186 is more closely
related to MTF134, than to MTF133, in terms of the lytic
function. Referring to Table 1 it can be seen that this

10   ability to give lysis correlates with the Arg 214 in the CH_1
domain and is independent of the residues found at position
356 and 358 in the CH_3 domain.

Figure 5 shows the results of an experiment following
on from the observations in Figure 4. The results shown,

15   confirms that it is the Arg at position 214 which is
necessary for the observed lysis in the aglycosylated IgG1
antibodies and that having the alternative residues of a
Lys or a Thr at this position does not give detectable
lytic activity.

20   The methods used herein are as described in
WO92/16562.

The data disclosed herein is based on the use of
different mutated γ1 heavy chain constant regions expressed
along with the heavy chain V-region and the light chain for

25   the CAMPATH-1 specificity (against antigen CDw52). The

vectors M13TG131 containing the γ1 gene (see Fig. 2), the
expression vector pSVgpt containing the V_R gene, methods of
mutation and methods of expression are all fully described
in WO92/16562.

5   The original human IgG1 "wild type" construct, YO
transfectant, clone TF57-19 was of immunoglobulin allotype
Glm(1,17). An alternative allele was constructed by
mutating the residues in CH_1 and CH_3 (using oligonucleotide
primers MO1 and MO2 as described in WO92/1652) to give the
10   allotype Glm(3), clone MTF121. In a second step, the
Glm(3) allotype was eliminated by replacing the Arg 214
with a Thr (using oligonucleotide primers MO2 and MO4 as
described in WO92/16562) to give the "null" allotype of
clone MTF123. Comparison of these constructs under optimal
15   conditions indicates that they were very similar in their
abilities to mediate autologous human complement dependent
lysis of human lymphocytes.

For these new experiments, some modifications to the
above described constructs have been made. The major and
20   starting modification is a further mutation in the CH_2
domain encoding intron to alter the conserved site of N-
linked glycosylation by mutating the Asn 297 to Ala. This
was achieved by site directed mutagenesis (as used
previously for the allotype mutants) of the appropriate
25   sequence using an oligonucleotide primer "MO3" with the

following sequence.

     5' CCG GTA CGT GCT AGC GTA CTG CTC CTC 3'

to give the final γ1 sequence of CH_2 of

297

Glu Glu Gln Tyr <u>Ala</u> Ser Thr Tyr Arg

5' GAG GAG CAG TAC GCT AGC ACG TAC CGG 3'

3' CTC CTC GTC ATG CGA TCG TGC ATG GCC 5'

The wild type γ1 Glm(1,17) sequence was mutated in the M13TG131 vector with oligonucleotide MO3. The sequence was checked and the constant region gene was recloned into the pSVpgt vector containing the CAMPATH-1 VH region and transfected into YO cells along with the light chain to give the expressed clone MTF133. In a similar way, the allele Glm(3) was mutated and expressed as clone MTF134 (see Table 1 for summary).

Supernatant from cultures of these two clones were firstly screened for production of human IgG1 antibody with CAMPATH-1H specificity using the Enzyme-Linked Immuno-Sorbent Assay (ELISA) method described in Example 1 of WO92/01652. Positive clones were grown up to bulk cultures and the antibody concentrated by precipitation with 50% saturated ammonium sulphate. Following dialysis into phosphate-buffered saline (PBS), the concentration of the antibodies were estimated by a quantitative ELISA. Complement dependent lysis was carried out as described in

21

Example 2 of WO92/16562. Human peripheral blood
mononuclear cells were labelled with ^{51}Cr, the cells were
added to titrations of the antibodies and human serum was
added as a source of complement. The percentage of

5   specific ^{51}Cr release was calculated by measuring released
cpm after a 1 hour incubation at 37°C. From the literature
and their own studies with an aglycosyl γ1 antibody to
human CD3 [Bolt, S., et al (1993) Eur. J. Immunol. 23, p
403-411 The generation of a humanised, non-mitogenic CD3

10   monoclonal antibody which retains in vitro
immunosuppressive properties] and another to mouse CD8
[Isaacs, J. D., et al (1992) Immunol. 148, p 3062-3071
Therapy with monoclonal antibodies - an in vivo model for
the assessment of therapeutic potential], the present

15   applicants had fully expected the aglycosyl mutants to be
equally defective in complement mediated lysis compared to
the wild type glycosylated γ1 antibody. To their surprise
they found that unlike the Glm(1,17) aglycosyl antibody
MTF133 the Glm(3) aglycosyl antibody MTF134 had some

20   residual lytic ability (see Figure 3). The relevant amino
acid residues for the two antibodies MTF133 and MTF134 are
shown below.

     <u>Name</u>   <u>Allotype</u>   <u>Phenotype</u>   <u>Amino Acids</u>

25   <u>214</u> <u>297</u> <u>355</u> <u>356</u> <u>357</u> <u>358</u>

MTF133   Glm(1,17)   Aglycosylated   Lys Ala Arg Asp Glu Leu

MTF134   Glm(3)   Aglycosylated   Arg Ala Arg Glu Glu Met

As these two antibodies differ from each other only in the Arg/Lys at 214 in the CH_1 domain and the Glu/ASP at 356 and in the Met/Leu at 358 in the CH_3 domain, it became of interest to resolve what was the crucial difference responsible for the observations, particularly because all previous studies have tended to indicate that it is the CH_2 domain which is crucially important for complement dependent lysis [eg Greenwood, J.D., et al (1993) Eur. J. Immunol. 23 p 1098-1104 Structural motifs involved in human IgG antibody effector functions and Greenwood, J.D., and Clark. M.R., in: Protein engineering of antibody molecules for prophylactic and therapeutic applications in man (ed. Clark M.R.) Pub. Academic Titles, UK (in press 1993) Effector functions of matched sets of recombinant IgG subclass antibodies] and the above result indicates a possible role for the CH_1 and/or CH_3 sequences.

To resolve this, the present applicants prepared some more antibody constructs. Using a PstI restriction fragment from the M13TG131 γ1 containing vectors, it is possible to remove the CH_1 encoding exon complete from one construct and to swap it with the homologous fragment from another (see Figure 2). In this way, the CH_1 domains were

23

swapped and expressed clones were made in which Arg 214 was
present with Asp 356, Leu 358 (clone BTF186) and another in
which Lys 214 was present with Glu 356, Met 358 (clone
BTF187) (see summary in Table 1). A third CH_1 swap mutant

5   was constructed in which the "null" allotype Thr 214 was
present with Asp 356, Leu 358 (clone BTF188). The relevant
amino acid residues for the three new antibody constructs
BTF186, BTF187 and BTF188 are shown below.

10   Name Allotype Phenotype   Amino Acids

                                            214 297 355 356 357 358

     BTF186   Glm(1,3)   Aglycosylated   Arg Ala Arg Asp Glu Leu

     BTF187   Glm(17)   Aglycosylated   Lys Ala Arg Glu Glu Met

     BTF188   Null at   Aglycosylated   Thr Ala Arg Asp Glu Leu
15   214

     All three of these new constructs were recloned into
pSVgpt, expressed and tested by ELISA and by complement
assay as above. The results (see Figures 4 and 5) indicate

20   quite clearly that it is the Arg 214 difference in the CH_1
which correlates with the observed functional difference.
The applicants have found that the results are highly
reproducible and are consistent with three different donors
of the cells and complement being used.

25   Enzyme linked immunoadsorbent assays were used to
allotype the secreted antibodies generated by the genetic

24

constructs and these confirm the presence of the expected

allotypes (including the revised data for BTF188). The

assays were adapted from the methods described in Cobbold,

S.P., et al., Meth. Enzymol. 1990 127, p 19-24 and were

5   carried out as described briefly below.

Monoclonal antibodies specific for the three Glm

allotypes 1, 3 and 17 (also known as a, f and z

respectively) were obtained from the Central Laboratory of

the Netherlands Red Cross Blood Transfusions Service

10   (Plesmanlaan 125, 1066 CX Amsterdam)

Code	Specificity	Clone	Batch
R2102	anti-Glm(a)	5E7	MG102-P1
R2104	anti-Glm(f)	5F10	MG104-P1
R2101	anti-Glm(z)	5A1	MG101-P1

15

These were used to coat the surface of the wells of

three microtitre plates using a volume of $50\mu l$/well and a

protein concentration of $10\mu g$/ml. The plates were then

washed with phosphate buffered saline (PBS) containing 0.1%

20   w/v of Bovine Serum Albumen (BSA) and were then blocked

with PBS 1% BSA. The antibodies to be tested were

resuspended in PBS to approximately $10-100\mu g$/ml and were

dispensed onto the three different coated plates in

duplicate wells. After 1 hours incubation, the plates were

25   again washed with PBS 0.1% BSA and a biotinylated

25

monoclonal antibody (NH3/41.34) specific for human κ light

chains was added at a concentration of 10μg/ml as a

detection reagent. Bound biotinylated antibody was

detected with subsequent steps of streptavidin peroxidase

5   followed by the substrate o-phenylenediamine in citrate-

phosphate buffer. The conversion of substrate was

determined by measuring the absorbance at 495nm. Results

from the three assays were combined in Table 2.

The functional differences identified as existing by

10   the present applicants are likely to be of importance

depending upon the therapeutic use of a given antibody.

Firstly it seems reasonable to assume that the trends in

the functional differences seen here would also be true for

other specificities than just CAMPATH-1H, although the

15   effects may be of a different magnitude in plateau and

titre. Secondly it is also likely that although it was

necessary to make the aglycosyl mutants to observe these

differences with CAMPATH-1 specific antibodies, the

functional differences might still be there, but less easy

20   to see under optimal conditions with glycosylated

antibodies (CAMPATH-1 is noted as an exceptionally good

target for complement dependent lysis). Thus in situations

where it is important to have an antibody with the best

activity in complement lysis it may be appropriate to use

25   the Arg 214 variants as in allotype Glm(3). Alternatively

in situations where no effector functions are required, as
in the example of the non-mitogenic CD3 antibody, then a
mutant with Lys or Thr at 214 combined with a conversion of
asparagine at position 297 to alanine or some other amino
acid would be appropriate.

These results indicate a role for the CH_1 in activation
of complement and also point at possible functional
differences in immunoglobulin alleles which might become
apparent under some experimental conditions.

From all the above it can be seen that some of the
residues which differ between two alleles of the same
subclass are in some cases identical with the residues
found in a similar position in a different subclass.

For example in the CH_1 at position 214, arginine is
found in IgG4 and IgG3 whilst threonine is found in IgG2.
In IgG1 the amino acid at position 214 depends on the
allotype, such that arginine is found in the allotype
(G1m(3) and lysine is found in the allotype G1m(17). Thus
it follows that in this region the sequence of the allotype
G1M(3) is more similar to IgG3 and IgG4 than is the
allotype G1m(17). In the work reported herein, the
applicants have shown that the IgG1 antibody with an
arginine at position 214 (G1m(3)) is more active in
complement activation that the IgG1 antibody with a lysine
at position 214 (G1m(17)). Putting this observation

together with the homology between subclasses, it seems
reasonable to propose that the arginine at position 214 in
IgG3 may play some important role in moderating the
function of that other subclass. [This however does not

5   preclude the possibility that some additional amino acid
changes at a different site may serve to counteract the
effect for example the changes in the CH_2 domain of IgG4
which completely abolish complement activation]. In
support of this is the observation that an aglycosyl IgG3

10   antibody (Lund et al 1990 Mol. Immunol. 27, 1145-1153) has
some residual activity in complement activation. It seems
reasonable therefore, to identify the arginine at 214 as a
likely candidate for causing the observed difference
between aglycosyl IgG3 and aglycosyl IgG1 G1m(17). Similar

15   arguments can be extended to other allotypes of the four
subclasses in which the residues present in one allotype of
one subclass are identical to residues found at that
position in another subclass and may therefore contribute
to the observed functional differences between subclasses.

20   To test this, the skilled person could easily extend the
approach described here for IgG1 allotypes to other
subclasses ie make different mutants in which only some of
the allelic residues are altered at any one time and see if
any of these correlate with functional changes between

25   mutants.

Thus it follows from the above, that one can moderate the functions of one subclass by introducing mutations at positions homologous to given alleles of another subclass, ie if the arginine at position 214 in IgG3 were changed to a lysine (found in G1m(17) but not usually in IgG3), then the activity in complement activation of the mutant IgG3 should be reduced compared to wild type IgG3.

Another important point illustrated by the work described herein is the identification of residues in the CH_1 which seem to affect complement activation. Previous work has always tended to identify the much stronger effects due to differences in the CH_2 domain. Thus there is functional significance to allotypic residues in all parts of the antibody not just the CH_2.

The foregoing teaches that allotypic polymorphism is to a certain extent responsible for different effector functions. Thus it allows the creation of binding molecules having a heavy chain which predisposes the molecule to a particular effector function.

It will be possible to target immune effector mechanisms to various cell types by attaching different molecules to the N terminal of an IgFc fragment (comprising a pair of second amino acid sequences each comprising part or all of a human immunoglobulin heavy chain having an allotypic determinant of a sequence associated with a

desired effector function) and using the specificity of the
attached molecule to label particular cells for
destruction. For instance, attachment of a soluble form of
a transmembrane receptor, coding for the extracellular

5   portion of that receptor, to the IgFc would produce a
molecule capable of binding to a cell expressing a ligand
for the receptor (eg binding of soluble CD40.Fc to cells
expressing the CD40 ligand, Armitage et al (1992), Nature
347: 80-82).

10   Alternatively, one could attach a cytokine to the IgFc
fragment and use that hybrid molecule to label cells
expressing the receptor for the cytokine (eg binding of IL-
2/IgG1 chimeric molecule to cells expressing IL-2
receptors, Landolfi (1991), J. Immunol. 146: 915-919).

15   This attachment to IgFc may be by chemical linkage or by
recombinant DNA technology to produce a fusion protein.

Cells thus labelled could then be destroyed via immune
effector activation directed by the IgFc portion of the
fusion protein.

20

Table 1　Summary of expressed clones and their characteristics

Clone Name	M13 Construct	Constant Region	Phenotype	Allotype	214	Position 297	356	358	Oligos Used
TF57-19	G1 w.t.	γ1 wild type	fully wild type	G1m(1,17)	Lys	Asn	Asp	Leu	None
MTF121	G1M1M2	γ1 mutant	γ1 allele	G1m(3)	Arg	Asn	Glu	Met	M01.M02
MTF123	G1M2M4	γ1 mutant	γ1 "null" allele	"null"	Thr	Asn	Glu	Met	M02.M04
MTF133	G1M3.	γ1 mutant	aglycosylated w.t.	G1m(1,17)	Lys	Ala	Asp	Leu	M03
MTF134	G1M1M2M3	γ1 mutant	aglycosylated allele	G1m(3)	Arg	Ala	Glu	Met	M01.M02.M03
BTF186	G1M1M3.	γ1 mutant	aglycosylated allele	G1m(1,3)	Arg	Ala	Asp	Leu	M01.M03
BTF187	G1M2M3	γ1 mutant	aglycosylated allele	G1m(17)	Lys	Ala	Glu	Met	M02.M03
BTF188	G1M3M4	γ1 mutant	aglycosylated "null"	"null",G1m(1)	Thr	Ala	Asp	Met	M03.M04

"Clone Name" gives the name given to the final expressed product in Y0 cells transfected with a pSVgptV$_H$CAMPATH construct. The "M13 Construct" is the name of the intermediate M13TG131 construct used in derivation of the final antibody. Also given is information on the expressed constant region phenotype and the allotype of the antibodies together with the amino acids found at the relevant positions in the γ1 sequence. The final column gives details of the mutagenic oligonucleotides used in for the derivation of the construct.

Table 2   Enzyme linked immunoadsorbent assay to detect immunoglobulin allotypes of transfectants

Table 2a

		MTF121	MTF123	MTF133	MTF134	BTF186	BTF187	BTF188	TF57-19	CNTRL
Anti Glm(a)	Anti Glm(1)	0.33	0.39	1.08	0.31	0.89	0.61	0.99	1.55	0.13
Anti Glm(f)	Anti G1M(3)	0.82	0.39	0.31	0.95	0.82	0.49	0.34	0.31	0.31
Anti Glm(z)	Anti Glm(17)	0.63	0.83	1.61	0.56	0.72	1.55	0.52	1.89	0.21

Table 2b

		MTF121	MTF123	MTF133	MTF134	BTF186	BTF187	BTF188	TF57-19	CNTRL
Anti Glm(a)	Anti Glm(1)	0.14	0.18	0.67	0.13	0.53	0.33	0.61	1.00	0.00
Anti Glm(f)	Anti G1M(3)	0.80	0.12	0.01	1.00	0.80	0.28	0.05	0.00	0.00
Anti Glm(z)	Anti Glm(17)	0.25	0.37	0.83	0.21	0.30	0.80	0.19	1.00	0.00

Table 2c

		MTF121	MTF123	MTF133	MTF134	BTF186	BTF187	BTF188	TF57-19	CNTRL
Anti Glm(a)	Anti Glm(1)	-	-	+	-	+	-	+	+	
Anti Glm(f)	Anti G1M(3)	+	-	-	+	+	-	-	-	
Anti Glm(z)	Anti Glm(17)	-	-	+	-	-	+	-	+	

The results of three ELISA assays to detect the allotypes Glm(1), Glm(3) and Glm(17) are summarised above. In 2a the results are given as the average of duplicate wells in terms of the measured absorbance at 495nm. For convenience of comparison between the three assays these results are then normalised in table 2b to a scale of 0 (lowest value) to 1 (highest value). Finally in 2c the results are interpreted in terms of a positive result being greater than 0.5 and a negative less than 0.5. The results confirm the expected allotypes predicted from the genetic constructs detailed in Table 1. The allotypes are also given in terms of the two recognised systems of notation ie in both their numeric and the equivalent alphabetical numenclature a=1, f=3, z=17.

CLAIMS

1. A binding molecule which has

    a first amino acid sequence comprising a domain with

    an ability to bind to a target molecule; and

    a second amino acid sequence substantially homologous

    to part or all of the constant region of a human

    immunoglobulin heavy chain, but which differs in an

    allotypic determinant

    wherein the difference in the allotypic determinant

    results in said binding molecule having an improved

    effector function as compared to a binding molecule having

    the first amino acid sequence and part or all of the

    constant region of the immunoglobulin heavy chain.

2. A binding molecule according to claim 1 wherein the
    domain comprises an antibody binding domain.

3. A binding molecule according to claim 2 wherein the
    antibody binding domain is non-human.

4. A binding molecule according to claim 3 wherein the
    antibody binding domain is of rodent origin.

5. A binding molecule according to claim 1 wherein the
    domain comprises part or all of a cytokine.

6. A binding molecule acc rding to claim 5 wherein the

33

cytokine comprises anyone of IL-2, IL-4, IL-5, IL-8, IL-12 IL-13 and IL-15.

7.   A binding molecule according to claim 6 wherein cytokine comprises IL-12.

8.   A binding molecule according to claim 1 wherein the domain comprises part or all of a soluble form of a cell bound ligand or a cell bound receptor.

9.   A binding molecule according to claim 8 wherein the cell bound receptor comprises the OX-40 receptor.

10.   A binding molecule according to claim 8 wherein the cell bound ligand comprises the OX-40 ligand.

11.   A binding molecule according to any one of claims 1 to 10 wherein the human immunoglobulin heavy chain is of the isotype IgG1.

12.   A binding molecule according to any one of claims 1 to 11 wherein the difference in an allotypic determinant is in the CH_1 domain.

13.   A binding molecule according to any one of claims 1 to 12 wherein the difference in an allotypic determinant comprises the presence or absence of arginine at position 214.

14. A binding molecule according to any one of claims 1 to 11 wherein the difference in an allotypic determinant is in the CH_2 domain.

5   15. A binding molecule according to claim 14 wherein the difference in an allotypic determinant comprises the presence or absence of asparagine at position 297.

16. A binding molecule according to any one of claims 1 to

10   15 wherein the second amino acid sequence differs from the part or all of the constant region of an immunoglobulin heavy chain in more than one allotypic determinant.

17. A binding molecule according to claim 16 wherein the

15   differences are in both the CH_1 and CH_2 domains.

18. A binding molecule according to claim 17 which comprises an amino acid other than arginine at position 214 of the CH_1 domain and an amino acid other than asparagine at

20   position 297 of the CH_2 domain.

19. A pharmaceutical preparation which comprises a binding molecule according to any one of claims 1 to 18.

25   20. A reagent which comprises a binding molecule according to any one of claims 1 to 18.

21. A recombinant construct which comprises a nucleotide

sequence coding for a binding molecule according to any one
of claims 1 to 18.

22.   A cloning or expression vector which comprises a
construct according to claim 21.

23.   A host cell which comprises a vector according to
claim 22.

24.   A transgenic animal which carries a recombinant
construct according to claim 21.

25.   A method for making a binding molecule which has a
first amino acid sequence comprising a domain with an
ability to bind to a target molecule and a second amino
acid sequence comprising part or all of a human
immunoglobulin heavy chain having an allotypic determinant
of a sequence associated with a desired effector function
which method comprises:

     (a)   examining one or more sequences, each comprising
part or all of a human immunoglobulin heavy chain and
obtaining said second amino acid sequence by either (i)
selecting one of said one or more sequences on the basis of
it comprising a said allotypic determinant; or (ii) by
selecting one of said one or more sequences on the basis of
it comprising a sequence suitable for altering to a said
allotypic determinant and altering the sequence to make a
said second amino acid sequence;

(b)   obtaining a said first amino acid sequence;

(c)   creating the binding molecule by either (i) chemically linking said first and second amino acid sequences; or (ii) making a nucleotide construct encoding said first and second amino acid sequences, transforming a suitable host cell with the nucleotide construct, and causing said host cell to express the binding molecule in the form of a fusion polypeptide.

26.   A method according to claim 25 wherein the domain is an antibody binding domain.

27.   A method according to claim 26 wherein the antibody binding domain is non-human.

28.   A method according to claim 27 wherein the antibody binding domain is of rodent origin.

29.   A method according to claim 25 wherein the domain comprises part or all of a cytokine.

30.   A method according to claim 29 wherein the cytokine comprises any one of IL-2, IL-4, IL-5, IL-8, IL-12, IL-13 and IL-15.

31.   A method according to claim 30 wherein the cytokine comprises IL-12.

32.   A method according to claim 25 wherein the domain comprises part or all of a soluble form of a cell bound ligand or a cell bound receptor.

5   33.   A method according to claim 32 wherein the cell bound receptor comprises the OX-40 receptor.

34.   A method according to claim 32 wherein the cell bound ligand comprises the OX-40 ligand.

10

35.   A method according to claim 25 wherein human immunoglobulin heavy chain is of the isotype IgG1.

36.   A method according to any one of claims 25 to 35

15   wherein the binding molecule comprises an amino acid other than arginine at position 214 of the CH_1 domain of the human immunoglobulin heavy chain and/or an amino acid other than asparagine at position 297 of the CH_2 domain of the immunoglobulin heavy chain.

20

38.   A method of treating a patient which comprises administering a pharmaceutical according to claim 19.
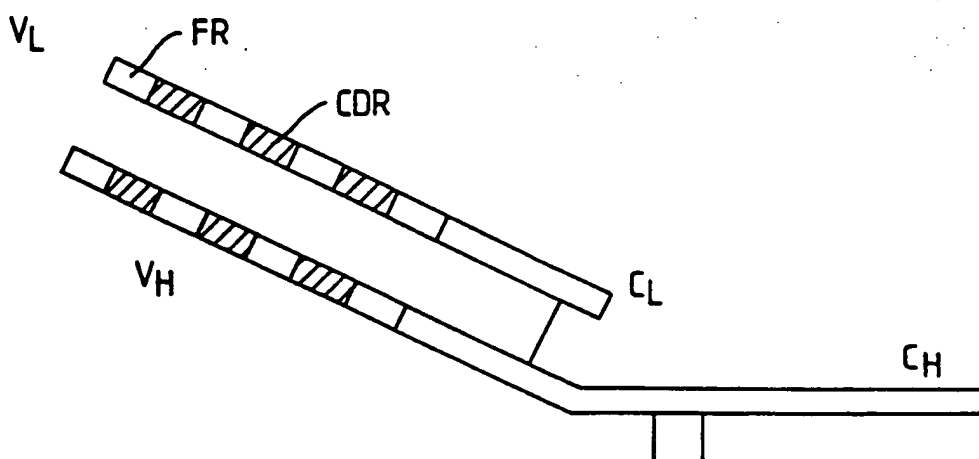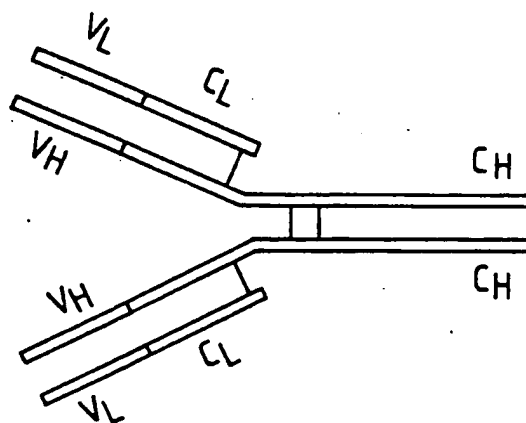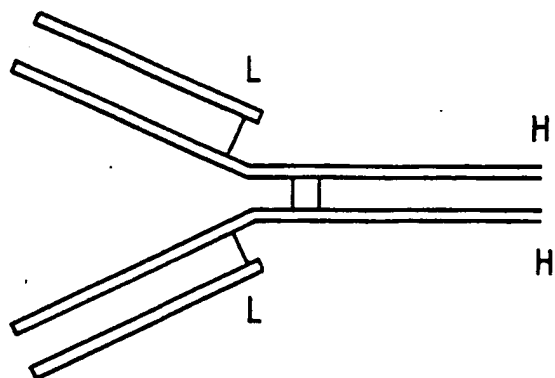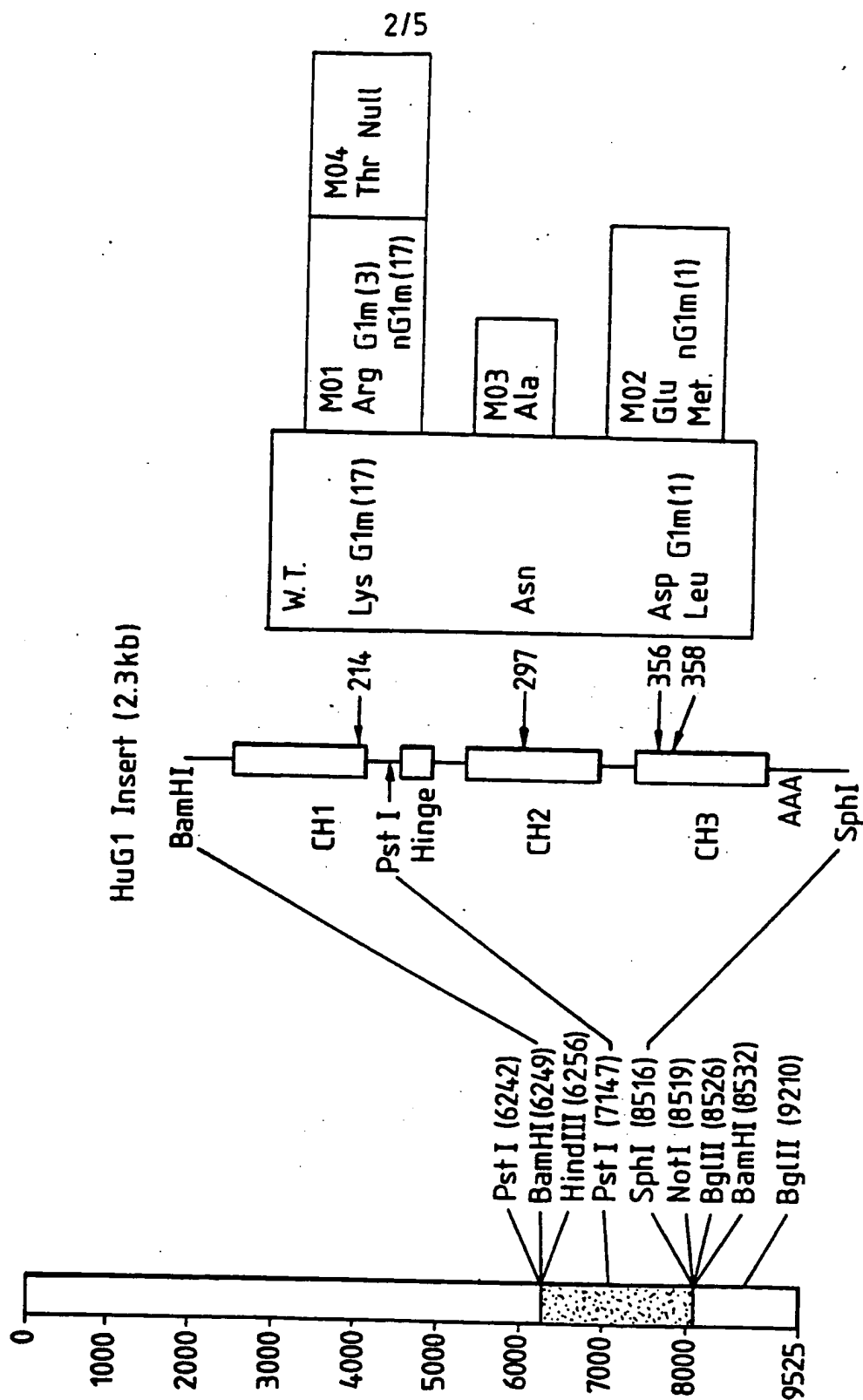
Fig.1.
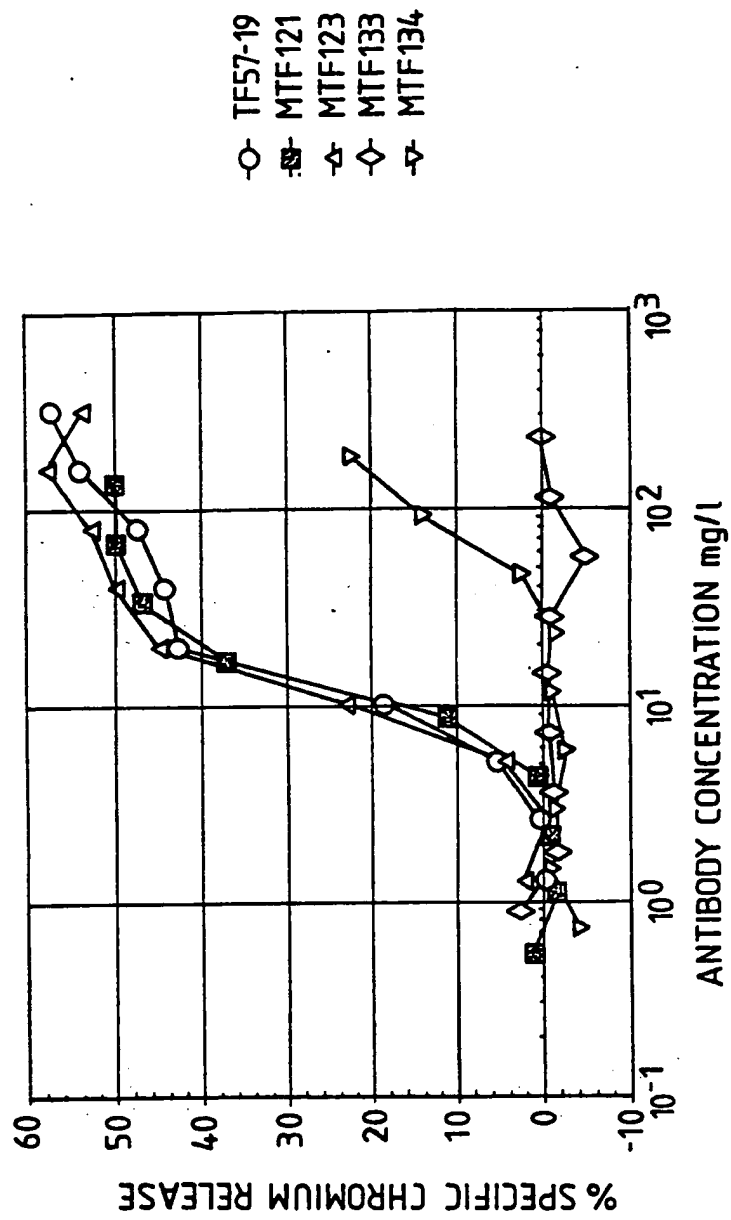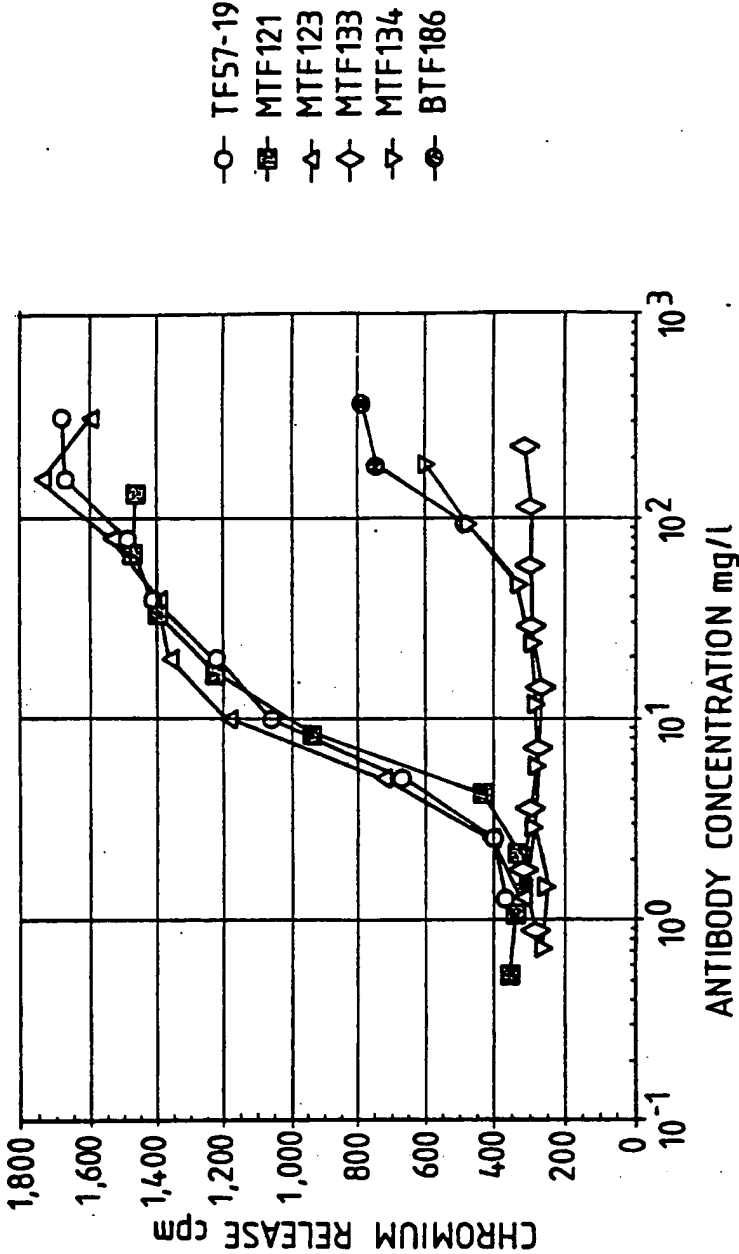
Fig.2.

2/5

3/5

Fig.3.

4/5

Fig.4.

○	TF57-19
■	MTF121
△	MTF123
◇	MTF133
▽	MTF134
●	BTF186

CHROMIUM RELEASE cpm

ANTIBODY CONCENTRATION mg/l

Fig.5.

TF57-19 —○—
BTF186 —■—
BTF187 —△—
BTF188 —◇—

% SPECIFIC CHROMIUM RELEASE

ANTIBODY CONCENTRATION mg/l
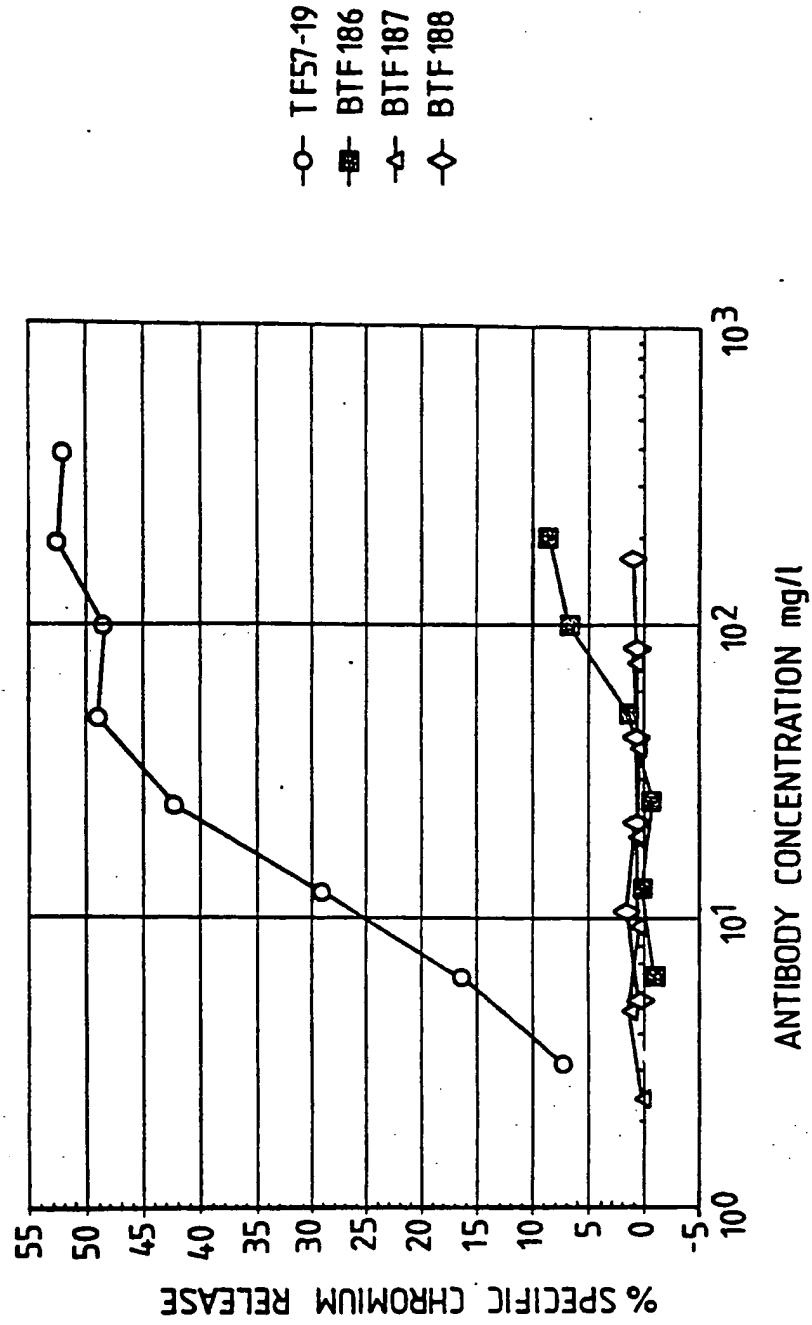
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6   C12N15/62   C07K19/00   C07K14/54   C07K14/55   C07K14/705
         A61K39/395   C07K16/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12N   C07K   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,92 16562 (LYNXVALE LTD, GB) 1 October 1992 cited in the application	1-4,8, 11-23, 25-28, 32,35-38
Y	see the whole document	5,6,9, 10,29,30
Y	WO,A,92 08495 (ABBOTT BIOTECH, INC, US) 29 May 1992 see page 11, line 14 - page 20, line 1; examples 2,3	5,6,29, 30

-/--

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
9 December 1994	22. 12. 94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Nauche, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.17B, February 1993 page 73 BIRKELAND, M. ET AL.; 'Cloning and expression of mouse OX-40 the homologue of a rat cell surface protein restricited to CD4-posisitve T blasts' & Keystone symposisum on cytokines and cytokine receptors : from cloning to the clinic, Keystone; Colorado; USA; 31 january- 7 frbruary 1993 Abstract E204	·9,10
X	INTERNATIONAL REVIEW OF IMMUNOLOGY, vol.10, no.2-3, 1993, USA pages 241 - 250 JOFFLIFFE, LINDA K.; 'Humanized antibodies : enhancing therapeutic utility through antibody' see the whole document	1-4
X	BIOTECHNIQUE, vol.4, no.3, 1986 pages 214 - 221 OI, V.T.; MORRISON S.L.; 'Chimeric Antibodies' see page 215, column 2, line 29 - page 215, column 3, line 23	1-4

5

Box I   Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Remark: Altough claim 38 is directed to a method of treatment of the human/
   animal body as well as diagnostic methods, (Rule 39.1(iv)PCT) the search
   has been carried out and based on the alleged effects of the compound/
   composition.

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such
   an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II   Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all
   searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment
   of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report
   covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is
   restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

                               ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9216562	01-10-92	EP-A-	0575407	29-12-93
WO-A-9208495	29-05-92	AU-A-	9059691	11-06-92
		CA-A-	2095836	10-05-92
		EP-A-	0574395	22-12-93